TABLE 2. Preclinical findings for 638T, 638T transformed with pVT1, and 638 in mice and rabbits

Strain	Colonization[a] (no. of vibrios)		Immune response[b]			
			Vibriocidal antibody GMT (range) on day:		Anti- LPS IgG GMT (range) on day:	
	Inoculated	Recovered[a]	14	28	14	28
638	5.9×10^6	1.5×10^6	800 (320–2,500)	500 (320–1,200)	150 (100–300)	316 (200–400)
638T	1.0×10^6	3.0×10^5	485 (80–2,560)	368 (80–1,280)	355 (100–1,600)	848 (100–3,200)
638T/pVT1	3.6×10^6	1×10^6	640 (160–1,280)	320 (160–640)	640 (100–3,200)	640 (100–3,200)

[a] Each value represents the average for at least eight mice from two independent experiments.
[b] Each value represents the average for at least six rabbits. The day 0 titers were under 1:20 for vibriocidal antibodies and under 1:25 for anti-LPS IgG.

remained high up to day 28 (Table 2). Consistent with these results, high titers of anti-LPS IgG were induced by 638T at day 21 in all immunized rabbits (GMT = 479 [180 to 1,600]), with the peak observed at day 28 (GMT = 848 [100 to 3,200]). Among six rabbits, three seroconverted at day 7 and the other three seroconverted at day 21. Similar results were obtained with 638 and 638T/pVT1 (Table 2). No statistically significant differences could be detected in the titers of antibodies elicited by 638T and 638T/pVT1 or 638.

DISCUSSION

During this work, a DNA fragment containing the *thyA* gene from *V. cholerae* was cloned, sequenced, and demonstrated to contain two complete open reading frames, as represented in Fig. 1A. Bacterial TSs are highly conserved proteins, especially in regions involved in dUMP and folate binding (21). The predicted amino acid sequence encoded in *orf* 2 is shown above the corresponding coding sequence in Fig. 1B. Comparison of this sequence with those of proteins deposited in the Swiss-Prot database showed 78.4% identity with *H. influenzae* TS, 31.8% with *Neisseria gonorrhoeae* TS, and 31.4% with *E. coli* TS enzyme. According to the crystallographic data available on the stereochemical mechanism and structure of *E. coli* TS, the most important residues involved in substrate and cofactor binding have been established (21). Previous comparisons of TS amino acid sequences from different sources marked Arg-21, -126, -127, and -166; Glu-58; Trp-80; Tyr94 and -209; Leu-143 and -172; Cys-146; Ser-167; Asp-169; Gly-173; Phe-176; Asn-177; and His-207 as conserved residues implicated in substrate binding and catalytic function of the enzyme. For numbering, the TS of *E. coli* was taken as the reference. Except for Arg-127, *V. cholerae* TS is shown to contain all of the above residues in equivalent positions, as represented in Fig. 1B. These facts, together with the requirement for thymidine observed in several *orf* 2 mutants of *V. cholerae* constructed in our laboratory (results not shown), as well as the complementing ability of plasmids containing the complete *orf* 2, permit us conclude that *orf* 2 corresponds to the *thyA* structural gene of *V. cholerae*. In *E. coli*, *Igt* and *thyA* form an operon, and TS levels are regulated by transcription from the *Igt* promoter and by translational coupling due to the overlap of *Igt* and the ribosome-binding site of *thyA* (16). This overlap is also present in *V. cholerae* (Fig. 1B), and consequently, regulation by translational coupling seems plausible. The analysis of this region in the chromosome of 638 permitted us to design a procedure to mutate *thyA*, leaving intact the essential *Igt* gene, and obtain a viable mutant of *V. cholerae* by allelic replacement.

Biological containment is a desired feature of live cholera vaccines. Thymine or thymidine auxotrophy has been proposed as an environmental biosafety feature for environmentally released vaccines, since free pyrimidines are scarce in natural ecosystems (23). One important goal in developing the *thyA* mutant 638T was enhancement of the environmental safety of *V. cholerae* strain 638. We therefore evaluated the survival of 638T in minimal salts and in autoclaved and untreated water from different environmental sources in comparison to that of 638T transformed with pVT1. From our analysis, the following conclusions were derived. (i) Environmental survival in a culturable state of strain 638T is limited with respect to the control strain 638T complemented with pVT1 in autoclaved water from all sources tested. (ii) The survival ability of 638T is more limited in sewage and river water than in seawater. (iii) A sort of heat-sensitive factor(s), presumably biotic, exists in all environmental sources of water that limits the survival of *V. cholerae* in a culturable state and hampered comparisons in untreated water.

Previous reports demonstrated that LB-grown wild-type vibrios achieved the viable but nonculturable state at 20 days of incubation in a carbon source-free minimal medium (14). During our studies, the control strain survived at high counts for more than 20 days in autoclaved water from different sources. While this is the case for the control strain 638T/pVT1, the 638T counts became undetectable after 11 and 18 days in sewage and river water, respectively. These results point to a significant contribution of the wild-type *thyA* gene to the survival of *V. cholerae* and reinforce our proposal to use 638T as a cholera vaccine prototype with enhanced environmental safety features. The greater persistence of 638T in seawater than in the rest of the waters tested remains unexplained, but it is highly encouraging that in the more immediate human environment, 638T has diminished survival with respect to the control strain. This is even more important since it was recently demonstrated that the hemagglutinin protease of *V. cholerae* is capable of proteolytic inactivation of CTXΦ (18). As in 638T, the HA/P coding gene is inactivated; this strain seems more vulnerable to infection by CTXΦ. However, in addition to the immunity conferred by the remnant RS1 in 638T to reacquisition of this phage, the *thyA* mutation makes superfluous this and most of the other means of acquisition of genetic information.

Although 638T displayed limited survival in the conditions tested, it was not at the expense of its colonization properties. Conclusions drawn from previous experiments on the relevance of *thyA* for colonization of the small bowel were debatable. *V. cholerae* CVD102, a spontaneous thymine-dependent auxotroph of CVD101 (*ctxA ctxB*$^+$ live oral cholera vaccine candidate), was studied in volunteers. This strain showed diminished immunogenicity and colonization potential with respect to CVD101, which suggested that thymine auxotrophy was overattenuating (19). Later studies in the infant mouse cholera model showed that the reduced colonizing potential of this strain could not be compensated for with a functional *thyA* gene (2). Additionally, CVD102 showed reduced synthesis of